THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

IV. ON THE NATURE OF THE SPECIFIC POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS.

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It was shown in 1917 by Dochez and Avery (1) that there is present in fluid cultures of pneumococci a substance which precipitates specifically in antipneumococcus serum of the homologous type. This "soluble specific substance" was also found in the body fluids of the infected organism, and was even demonstrated in the urine of many patients suffering from pneumonia due to pneumococci of Types I, II, and III. Dochez and Avery also found that the specific substance is not destroyed by boiling, that it is readily soluble in water and precipitable from it by alcohol or acetone, that it does not dialyze through parchment, and that its serological reactions are unaffected by digestion with trypsin.

In later publications from this laboratory (2–4) it has been shown not only that this soluble specific substance appears to belong to the group of polysaccharides, but also that it is possible to isolate from cultures of each antigenic type of Pneumococcus a chemically distinct polysaccharide, different in many particulars from those of the other two types, and possessing in the highest degree the property of precipitating the antiserum to the type of Pneumococcus from which it was derived. Analogous sugar derivatives with specific properties have been isolated in the case of the Friedländer bacillus (Heidelberger, Goebel, and Avery (5), Mueller, Smith, and Litarczek (6)), the tubercle bacillus (Laidlaw and Dudley (7), Mueller (8)), and even from yeast (Mueller and Tomcsik (9)), and their presence has been shown in *Streptococcus viridans* (Lancefield (10)). The so called "residue

antigens" of Zinsser and Parker (11), first considered as containing in part protein degradation products or nucleic acid derivatives (12), appear also to be specifically reacting polysaccharides.

The immunological significance of this new type of sugar derivative, so frequently encountered among microorganisms, has been discussed elsewhere in papers from this and other laboratories (13–16), and its importance in the study of bacterial specificity demonstrated (17–19). It would accordingly be a matter of great interest to have some knowledge of the chemical structure of the new group of polysaccharides, and the present paper describes a beginning made in the case of the soluble specific substance of Type III pneumococcus.

As previously found (4), this substance may be obtained as a snow-white, amorphous powder, free from nitrogen and ash, and with marked acidic properties. It is precipitated from aqueous solution by strong mineral acids, and is then insoluble in water when dried. An aqueous solution of the sodium salt shows a specific rotation of about $-34°$ and gives precipitates with many heavy metal salts and with barium hydroxide in excess. At a dilution as high as 1 to 6,000,000 it still yields a detectable precipitate with Type III antipneumococcus serum. The specific property disappears when the substance is hydrolyzed by means of acid. The products of hydrolysis are glucose and an acid which was considered to be a condensation product of a hexose and a -uronic acid.

The chief stumbling block to chemical progress with the bacterial polysaccharides has been the small amount of material available for investigation. It has been found possible in the case of the Type III pneumococcus to overcome this objection to some extent. In the first place this microbe produces far more specific substance than do the other two antigenic types of Pneumococcus, and in addition its polysaccharide is the easiest of the three to isolate and purify. Furthermore, once the properties of the Type III specific substance had been established, it was considered proper to add glucose to the usual Pneumococcus broth (2). In this way a greatly increased growth was obtained, and whereas the yields of specific substance were originally from 6 to 9 gm. per 300 liters of broth, they now rose to 35 to 40 gm. With these increased amounts of material it has been possible to show that the complex

sugar acid mentioned above is not only the chief product of hydrolysis, but that it is apparently the actual unit from which the whole polysaccharide is built up. The structure of the acid has also been partly elucidated.

EXPERIMENTAL.

A. Preparation of the Type III Soluble Specific Substance.

For the preparation of the specific polysaccharide the Type III pneumococcus was grown and the specific substance isolated as in Paper III (4), except that by the addition of 0.3 per cent of glucose to the liquid medium

TABLE I.

Preparation No.	Acid equivalent.	Specific rotation.	Total N.	Reducing sugars on acid hydrolysis.	Ash.	Precipitation with antipneumococcus serum.
33*	343	−30.5°	0.0	73.0	0.0	1:6,000,000
53†	338	−34.0°	0.0	70.0	0.0	1:6,000,000
55†	338	−33.0°	0.0	65.0	0.0	1:6,000,000

* Preparation 33 was isolated from glucose-free broth.

† Preparations 53 and 55 were obtained from the glucose broth.

far greater growth was obtained. Consequently the yield of purified polysaccharide was greatly improved, 300 liters of broth now furnishing between 35 and 40 gm. The material obtained was identical with that previously described, as will be seen from Table I.

B. Hydrolytic Products of the Type III Soluble Specific Substance.

1. Products of Partial Hydrolysis.—10 gm. of air-dried specific polysaccharide were dissolved in 40 cc. of 75 per cent (by weight) sulfuric acid at 0°. After standing for 21 hours in the ice box the solution was poured into 1 liter of water and the sulfuric acid quantitatively removed with barium hydroxide. The resulting solution was concentrated *in vacuo*. The product was found to be divisible into three distinct fractions. Fraction I was obtained by precipitation from a volume of 50 cc. with a slight excess of barium hydroxide saturated at 60°. Fraction III was also obtained as a barium salt by treating the concentrated, barium-free supernatant liquid from Fraction I at a volume of 20 cc. with 3 volumes of the same barium hydroxide solution. Fraction II represents the supernatant liquid of Fraction III, from which the remaining organic matter was precipitated with basic lead acetate after the excess of barium hydroxide had been removed. The lead was of course removed by means of hydrogen sulfide.

It was found possible further to subdivide Fraction I by precipitating a

neutralized solution with 10 per cent copper sulfate. From the precipitate (I*a*), and the supernatant liquid (I*b*), the free acids were obtained by eliminating the copper as its sulfide, the sulfate as barium sulfate, and concentrating to dryness. A summary of the properties of these five fractions will be found in Table II.

None of these fractions reacted specifically with Type III antipneumococcus serum. Since no glucose was found it is evident that the initial hydrolytic products of the Type III specific substance consist of a mixture of sugar acids probably of varying molecular weight, but containing a similar bionic acid unit as shown by the figures for the acid equivalent.

On further hydrolysis with boiling dilute acid these fractions were hydrolyzed to the disaccharide acid (aldobionic acid) described in a later section.

TABLE II.

Fraction No.	Acid equivalent.	$[\alpha]_D$	Reduction (calculated as glucose).
			per cent
I	340*	−12.6°	12.5
I*a*	343	−11.0°	11.8
I*b*	310	−7.9°	17.2
II	340	+9.2°	20.1
III	340	−8.3°	31.0

*This value remained unchanged in the presence of excess N/14 NaOH on the water bath.

2. Products of Prolonged Hydrolysis.—22.5 gm. of polysaccharide (containing 6.5 per cent of water of hydration) were slowly added with stirring and occasional cooling to 100 cc. of 75 per cent (by weight) sulfuric acid. After complete solution had resulted the mixture was placed in the ice box overnight and was then diluted to 2.7 liters and boiled 5 hours under a reflux. The sulfuric acid was removed quantitatively with barium hydroxide and the barium sulfate washed free from reducing sugars. The combined filtrates were concentrated to 150 cc. *in vacuo* and treated with an excess of basic lead acetate solution. After standing overnight in the cold, the portion precipitated by lead (Fraction I) was filtered off. The filtrate (Fraction II) was treated with hydrogen sulfide, and after the lead sulfide had been washed free from reducing sugars the filtrate was concentrated *in vacuo* and a small remainder of sugar acid (Fraction III) was removed by treating with an excess of basic lead acetate. All three fractions were now freed from lead, and in each case the lead sulfide was washed until free from reducing sugars. After removal of the hydrogen sulfide the

amount of sugar in each fraction was determined by the Shaffer-Hartmann method (20).

Fraction II, which had previously been shown to yield only glucose (4), contained 2.0 gm. of the sugar. Fractions I and III showed 8.7 and 0.2 gm., respectively, calculated as glucose. Since the two latter fractions contain a sugar, as will be shown later, having only one-half the reducing power of glucose their weights are ascertained by doubling the glucose value. On adding the three amounts thus obtained it is seen that 19.8 gm. of reducing sugars are accounted for, or 94 per cent of the theory. The total weight should be $22.5 \times 0.935 = 21.0$ gm.

a. Properties of Fraction I.—A portion of Fraction I was evaporated repeatedly with water *in vacuo* to remove acetic acid and was then reprecipitated as its lead salt. This was freed from lead and evaporated to complete dryness, yielding a friable, amorphous product. This material, which forms by far the major portion of the hydrolytic products of the specific polysaccharide, has been described in a preceding paper (4).

The acid equivalent of the crude disaccharide was determined by titration. 36.2 mg. neutralized 5.04 cc. of N/50 NaOH. This gives an acid equivalent of 361; calculated for $C_{11}H_{19}O_{10}COOH$, 356. A micro sugar determination by the method of Shaffer and Hartmann (20) on a sample of 1.92 mg. gave a back titration of 12.68 cc. of N/200 $Na_2S_2O_3$. This corresponds to copper and glucose equivalents of 2.02 and 0.95 mg., respectively. Reduction 49.5 per cent, calculated as glucose.

0.2882 gm. made up to 15 cc. with H_2O, $l = 2$, α, $+0.30°$. $[\alpha]_D = +7.8°$.

b. Formation of a Morphine Salt.—0.7 gm. of the dry sugar obtained as above was dissolved in 8 cc. of water and morphine was added in excess. Since the salt could not be made to crystallize from solutions containing water it was evaporated to complete dryness over phosphorus pentoxide and taken up in a mixture of equal parts of absolute methyl and ethyl alcohols. A small amount of dark insoluble gum was filtered off. During prolonged standing in the ice box a crystalline product gradually separated. After recrystallization from the same solvent the salt melted at 153–156° and was difficultly soluble in the usual anhydrous solvents.

0.2154 gm. substance: 3.7 cc. N_2 at 23°, 760 mm.

0.1003 " " : 0.1968 gm. CO_2 and 0.0557 gm. H_2O.

Calculated for $C_{29}H_{39}O_{15}N$ ($C_{12}H_{20}O_{12} \cdot C_{17}H_{19}O_3N$). C 54.26 per cent, H 6.13 per cent, N 2.18 per cent.

Found. C 53.52 per cent, H 6.22 per cent, N 2.03 per cent.

0.3888 gm. diluted to 15 cc. with water gave, in a 2 dm. tube, a rotation of $-2.48°$, changing after 24 hours to $-2.80°$, where it remained constant.

$[\alpha]_D = -47.9°$, changing to $-54.0°$.

c. Properties of the Sugar Acid Recovered from the Morphine Salt.—The morphine salt as prepared above was decomposed by the addition of a slight excess of aqueous ammonia. The resulting crystalline morphine was filtered off and the solution containing the sugar acid was twice precipitated with basic lead acetate. The acid was obtained by evaporating the lead-free product to complete dryness. It formed a snow-white, amorphous residue.

0.1053 gm. diluted to 15 cc. with water gave a rotation of +0.14° in a 2 dm. tube. $[\alpha]_D = +10.0°$.

2 mg. of substance, analyzed by the micro method of Shaffer and Hartmann (20), gave a titration of 12.90 cc. of N/200 $Na_2S_2O_3$ (factor 0.9660, blank 19.90 cc.). Copper equivalent, 2.12 mg., glucose 0.99 mg.; reducing sugar calculated as glucose, 49.5 per cent.

0.0956 gm. substance: 0.1398 gm. CO_2 and 0.0494 gm. H_2O.

Calculated for $C_{12}H_{20}O_{12}$. C 40.45 per cent, H 5.66 per cent.
Found. " 39.85 " " " 5.77 " "

35.17 mg. of sugar neutralized 4.85 cc. of N/50 NaOH. Acid equivalent, calculated for $C_{11}H_{19}O_{10}COOH$, 356; found, 363.

Except for a possible slight difference in optical rotation the crude disaccharide acid corresponds very closely in its properties with the purified acid.

0.2640 gm. of substance, heated with 12 per cent hydrochloric acid and analyzed by a modification of Pervier and Gortner's method (21) required 5.75 cc. of 0.1 N $KBrO_3$, corresponding to 0.0276 gm. of furfural, or 10.5 per cent. Since glucuronic acid yields about one-third of the amount of furfural liberated by pentoses under corresponding treatment (22), this figure would correspond roughly to the presence in the molecule of about 50 per cent of an acid of the glucuronic type.

The substance has marked acidic properties. It has one-half the reducing power of glucose and gives a strong naphthoresorcinol test. The acid is appreciably soluble in hot ethyl and methyl alcohols, and in hot glacial acetic acid, but fails to dissolve in the other usual organic solvents. On further prolonged hydrolysis it yields glucose. It thus appears to be built up from glucose and a -uronic acid in such a way that one reducing group remains free, and hence differs from any non-nitrogenous, naturally occurring disaccharide derivative hitherto described. Of all known sugar derivatives, it would seem most closely related to desaminochondrosin, the nitrogen-free anhydro derivative of chondrosin, a component of chondroitin sulfuric acid, which, in turn, occurs in mucoproteins (23).

d. Oxidation of the Sugar by the Method of Willstätter and Schudel (24).—0.1450 gm. of the sugar acid (recovered from its morphine salt), when analyzed by the method of Willstätter and Schudel, reduced 8.14 cc. of N/10 iodine. Theoretically 1 cc. of N/10 iodine should oxidize 0.0178 gm. of the disaccharide acid. 8.14 cc. therefore correspond to 0.1448 gm. of disaccharide acid. The sugar is obviously quantitatively oxidized and its reducing group must be aldehydic in nature since this method is specific for aldoses. The substance may, therefore, be termed an aldobionic acid.

e. Oxidation of the Aldobionic Acid with Strong Nitric Acid.—1.5 gm. of specific polysaccharide were dissolved in 8 cc. of 75 per cent sulfuric acid at 0°. After standing overnight in the ice box the solution was diluted with water until the acid was of normal concentration and was boiled 5 hours under a reflux. The aldobionic acid was isolated over the lead salt as described above. At a volume of 4 cc. its aqueous solution was treated with 10 cc. of 1:1 nitric acid, allowed to stand 15 hours, boiled for 3 minutes, and then quickly evaporated on a large watch-glass over a boiling water bath. No formation of mucic acid could be observed, so the solution of the oxidation product, at a volume of 6 cc., was made strongly alkaline with 40 per cent potassium hydroxide and then acidified with an excess of glacial acetic acid. It was then seeded with a small crystal of potassium acid saccharate and allowed to stand at 0° for 24 hours. The crystals which had formed were filtered off, washed with a few drops of ice water, and dried. 0.20 gm. was obtained. The salt was recrystallized from 1 cc. of boiling water, 0.100 gm. of pure potassium acid saccharate being recovered.

0.0496 gm. dry substance: 0.0173 gm. K_2SO_4.

Calculated for $HOOC(CHOH)_4COOK$. K 15.75 per cent.

Found. K 15.65 per cent.

f. Hydrolysis of the Disaccharide with Acid.—1.0 gm. of the dry aldobionic acid was dissolved in 50 cc. of normal sulfuric acid and boiled 20 hours under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide and the filtrate concentrated *in vacuo* and boiled with norit. The clear colorless solution, at a volume of 30 cc., was treated with an excess of basic lead acetate to remove the unaltered aldobionic acid. The filtrate was treated with hydrogen sulfide, filtered, and concentrated to dryness *in vacuo*. The residue was diluted to 20 cc. An analysis by the Shaffer-Hartmann method showed it to contain 0.1980 gm. of reducing sugars calculated as glucose. In a 2 dm. tube the solution gave a rotation of +1.07°. $[\alpha]_D = +54.1°$. The remaining solution was treated with 3.5 mols of phenylhydrazine acetate and heated for 1 hour on the water bath. The entirely crystalline osazone which was formed was filtered off and washed with a few drops of methyl alcohol. The yield was 0.10 gm. The product melted at 203–204°. The initial specific rotation was −54.5°, mutarotating to −30° after 48 hours.

From the melting point of the osazone, its direction of mutarotation, and finally from the specific rotation of the sugar solution itself, it is justifiable to conclude that this product of the hydrolysis of the aldobionic acid is glucose, and that the hexose half of the molecule is, therefore, glucose. The other half of the molecule (the sugar acid) is largely destroyed by acid hydrolysis (*cf.* (22)). Whether the saccharic acid identified in the preceding section arises from oxidation of the glucose or of the -uronic acid, or both, cannot be stated as yet.

g. Oxidation of the Aldobionic Acid with Bromine.—2.4 gm. of the acid were dissolved in 50 cc. of water and to the solution were added 8 gm. of barium carbonate and 1 cc. of bromine. After 2 days 0.5 cc. more bromine was added. 4 days later the solution was filtered, the excess of bromine blown out with air, and the barium and hydrobromic acid removed quantitatively with sulfuric acid and silver sulfate. The resulting solution, freed from silver and sulfuric acid, showed the presence of 10 per cent of unaltered disaccharide by its reduction value. Thus 90 per cent of the aldobionic acid had been oxidized to the corresponding dibasic acid; *i.e.*, the free reducing group had been oxidized.

It was thought possible to hydrolyze the dibasic acid, but boiling with normal sulfuric acid showed only a slight increase in reducing sugars. It may be, of course, that hydrolysis actually took place with the subsequent decomposition of the aldehydic sugar acid. It has as yet been impossible to isolate in a state of purity either the oxidation product itself, or the products of its hydrolysis. That the -uronic acid portion of the molecule is still intact, however, is indicated by the fact that the oxidation product gives a strong naphthoresorcinol test.

DISCUSSION.

In the original communication on the soluble specific substance of Type III pneumococcus (3) it was pointed out that on hydrolysis a product with some of the properties of glucuronic acid was obtained. In a later paper (4) it was shown that glucose was one of the products of hydrolysis, while the other isolated corresponded not with glucuronic acid itself but with a more complex derivative of the glucuronic type, possibly consisting of glucuronic acid combined with a hexose.

The following is now presented as evidence, when considered collectively, that this portion of the hydrolytic products of the Type III specific substance, precipitable by basic lead acetate, is actually a compound of glucose and a hexose-uronic acid.

1. The reducing power of both the crude aud the purified anhydrous substance is 50 per cent of that of glucose.

2. The acid equivalent is found to be 363, while the value calculated for $C_{11}H_{19}O_{10}\cdot COOH$ is 356.

3. As an acid, the substance forms a morphine salt which can be crystallized and purified by recrystallization. The analysis of the salt gives values for carbon, hydrogen, and nitrogen checking closely with the theoretical. The purified acid, recovered from the salt, is scarcely different from the crude material.

4. On prolonged hydrolysis only a small amount of glucose, in addition to unhydrolyzed material, can be isolated, the acid half of the portion hydrolyzed apparently decomposing similarly to glucuronic acid.

5. The reducing group of the disaccharide acid is aldehydic, as shown by the fact that it may be quantitatively determined by the Willstätter-Schudel method. The substance also gives the color reaction with naphthoresorcinol characteristic of the glucuronic acid type.

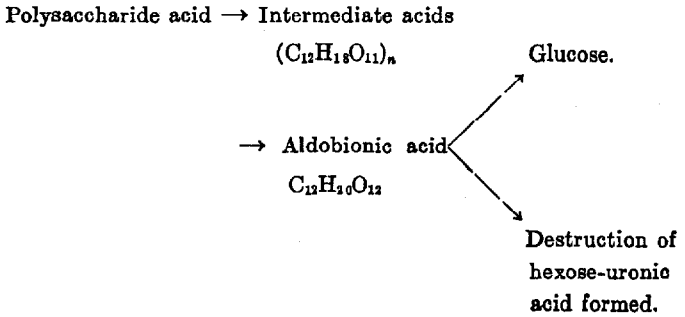
Since the easily isolable mucic acid is not formed on hydrolysis and oxidation with nitric acid, the acid portion of the molecule can scarcely be galacturonic acid. The saccharic acid actually recovered from the oxidation mixture certainly arises at least in part from the glucose fraction of the molecule; whether the acid portion takes part in its formation or gives rise to some other soluble acid must be left for future work to determine.

As to the position of the union of the glucose to the sugar acid, the evidence at hand does not permit any conclusion. The linkage may be either through the reducing group of the glucose, or through the reducing group of the sugar acid. That the union is glucosidic is indicated by the fact that on further hydrolysis the reducing power of the aldobionic acid increases to about 65 per cent before dropping owing to destruction of the hexose-uronic acid liberated.

If an analysis be made of the quantitative data obtained on the hydrolysis of the original polysaccharide (p. 617), it becomes evident that the aldobionic acid accounts for about 85 per cent of the total products of hydrolysis, while only 9.5 per cent is glucose, and 5.5 per cent unaccounted for. Thus by far the major portion of the polysaccharide seems to be constructed from molecules of this disaccharide acid. Now it has also been found (p. 619) that the aldobionic acid itself slowly hydrolyzes, to the extent of about 1 per cent per hour, on boiling with dilute mineral acid. It is therefore not illogical to assume that the 9.5 per cent of glucose liberated during the hydrolysis of the polysaccharide owes its origin, not to a separate part of the carbohydrate molecule, but chiefly to a secondary reaction involving the aldobionic acid. This assumption is all the more justified by the fact that no glucose is split off during the preliminary hydrolysis by 75 per cent

sulfuric acid in the cold. Since, also, these partial hydrolysis products show, by their acid equivalents, one carboxyl group for every two sugar nuclei, it would seem that the polysaccharide as a whole is built up of units of the aldobionic acid.

The condensed or polysaccharide form of a hexose-hexose-uronic acid should have the formula $(C_{12}H_{20}O_{12})_n - (n-1)H_2O$, or $(C_{12}H_{18}O_{11})_n$. A substance of this composition should have an acid equivalent of 338 and a carbon and hydrogen content of 42.6 per cent and 5.4 per cent respectively. These figures are practically identical with actual analytical values obtained ((4) p. 733, Preparation 33 II. Acid equivalent, 340; C, 42.7 per cent; H, 5.3 per cent). Thus one may justifiably conceive of the Type III soluble specific substance as a condensation product of the disaccharide acid, $C_{12}H_{20}O_{12}$, built up in such a way that the carboxyl groups remain free. Hydrolysis by means of acid follows the course:



In view of the evidence collected it is believed that the specific polysaccharide of the Type III pneumococcus is a definite chemical individual composed of units of a difficultly hydrolyzable aldobionic acid in which glucose and a hexose-uronic acid are combined in such a way that one aldehydic group and the carboxyl remain free. The polysaccharide is thus unusual not only in its possession of immunological specificity, but in its chemical constitution as well.

The question as to whether its unusual structure bears any relation to its immunological properties must be left for future work on this and other specific polysaccharides for a decision.

SUMMARY.

1. The soluble specific substance of Type III pneumococcus is shown to yield on hydrolysis a small amount of glucose and chiefly a disaccharide acid of a type not hitherto observed in any non-nitrogenous polysaccharide.

2. The disaccharide acid corresponds to the formula $C_{12}H_{20}O_{12}$ and contains one carboxyl group and one aldehydic reducing group in the molecule. It yields a crystalline morphine salt and appears to consist of 1 molecule of glucose condensed with 1 molecule of a hexose-uronic acid through one of the two reducing groups.

3. The specific polysaccharide is believed to be built up of units of this aldobionic acid, and thus to belong to a new type.

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